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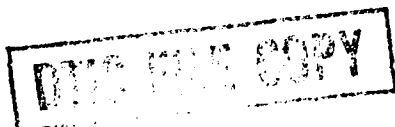
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obtained from animals following a superficial wounding. Cell extracts prepared from such regenerating epidermal cells have been found to be the richest source from all tissues tested.)

EDF activity was found to be destroyed in acid environment (pH 3), but stable in alkaline (pH 10), stable at 56°C but inactivated by 100°C. It was found to be stable when frozen at -20°C for many months and at 4°C for two weeks.

When separated by gel filtration chromatography the activity was found in three areas corresponding in molecular weight to 30, 5, and 1 kD.

Of different ion exchange columns only Q Sepharose was repeatedly shown to bind EDF(s) at pH 8 with high affinity. Elution was achieved with 0.5 M NaCl. Further purification of the factor was by FPLC Mono - Q ion exchanger and Reversed phase chromatography.

Thus, the best source of EDF was identified, a method of testing for its biological activity developed and steps necessary for biochemical purification established.

Toward the second objective - understanding the reasons for allogeneic epidermal cell graft rejection - we have found :

Induction of class II Major Histocompatibility antigens on keratinocytes is due to auto-induction by gamma interferon.

Gamma interferon production by keratinocytes is in response to allogeneic dermis.

Frozen viable dermis or trypsinized dermis has substantially diminished capacity to induce Ia antigens on keratinocytes and combination of freezing and trypsinization abolishes the dermal activity completely.

Fibroblasts, in contrast to keratinocytes do not respond by induction of class II antigens when exposed to allogeneic dermis, but Ia expression can be activated by exogenous gamma interferon.

Induction of class II antigens on human keratinocytes was shown by immuno-rosetting, induction of gamma interferon production by in situ hybridization using a gamma interferon probe and anti-gamma interferon sera were shown to inhibit induction of Ia antigens.

The studies suggest that keratinocytes are able to respond to allogeneic dermis, but not autologous, by induction of gamma interferon production. Subsequent auto induction of Ia antigens may contribute to recognition of allogeneic epidermal cells by T-lymphocytes and graft rejection.

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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SUMMARY

The studies described in this report were aimed at the biochemical characterization and purification of a wound healing growth factor, named Epidermal Cell derived Factor (EDF). The biological activity of EDF was found to embody enhancement of epidermal regeneration and regulation of dermal repair in skin wounds.

We have accomplished the following:

A tissue culture bioassay that is based on the capability of fibroblasts to contract collagen gels was shown to reflect *in vivo* effects of EDF on dermal cells and was adopted for examination of large numbers of samples during biochemical purification steps.

Supernatant fluids of epidermal cell cultures and cell extracts of epidermal cells grown in tissue culture were tested as possible sources for purification of the factor, but substituted by epidermal cells obtained from animals following a superficial wounding. Cell extracts prepared from such regenerating epidermal cells have been found to be the richest source from all tissues tested.

EDF activity was found to be destroyed in acid environment (pH3), but stable in alkaline (pH 10), stable at 56°C but inactivated by 100°C. It was found to be stable when frozen at -20°C for many months and at 4°C for two weeks.

When separated by gel filtration chromatography the activity was found in three areas corresponding in molecular weight to 30, 5, and 1 kD.

Of different ion exchange columns only Q Sepharose was repeatedly shown to bind EDF(s) at pH 8 with high affinity. Elution was achieved with 0.5 M NaCl. Further purification of the factor was by FPLC Mono - Q ion exchanger and Reversed phase chromatography.

Thus, the best source of EDF was identified, a method of testing for its biological activity developed and steps necessary for biochemical purification established.

Toward the second objective - understanding the reasons for allogeneic epidermal cell graft rejection - we have found :

Induction of class II Major Histocompatibility antigens on keratinocytes is due to auto-induction by gamma interferon.

Gamma interferon production by keratinocytes is in response to allogeneic dermis.

Frozen viable dermis or trypsinized dermis has substantially diminished capacity to induce Ia antigens on keratinocytes and combination of freezing and trypsinization abolishes the dermal activity completely.

Fibroblasts, in contrast to keratinocytes do not respond by induction of class II antigens when exposed to allogeneic dermis, but Ia expression can be activated by exogenous gamma interferon.

Induction of class II antigens on human keratinocytes was shown by immunorotetting, induction of gamma interferon production by in situ hybridization using a gamma interferon probe and anti- gamma interferon sera were shown to inhibit induction of Ia antigens.

The studies suggest that keratinocytes are able to respond to allogeneic dermis, but not autologous, by induction of gamma interferon production. Subsequent auto induction of Ia antigens may contribute to recognition of allogeneic epidermal cells by T-lymphocytes and graft rejection.

The research effort as outlined in our contract proposal had two major objectives:

- I) Investigate the role of epidermal cell derived growth factor(s) (EDF) in wound healing and compare it to other known growth factors and**
- II) Continue studies aimed at the understanding of allogeneic epidermal cell graft rejection.**

Our approaches and results toward accomplishment of these objectives are detailed below.

I. EPIDERMAL CELL DERIVED GROWTH FACTORS IN WOUND HEALING

EDF activity was discovered by us previously (1) in epidermal cell extracts and supernatant fluids of epidermal cell cultures. It was defined as an activity that enhances wound reepithelization by stimulating migration and proliferation of epidermal cells from hair follicles, glandular structures and wound edges. Moreover, as an inhibitor of migration/proliferation of fibroblasts in partial thickness surgically created wounds. When translated into its effects on cells in tissue culture it was shown to enhance proliferation of a subpopulation of epidermal cells and to prevent collagen shrinkage by fibroblasts. Hence, this activity was considered to play an important role in the wound healing process, particularly as a factor that might regulate scar tissue formation. To further our knowledge about the biological effects of a factor(s) that is responsible for the activity described, it was necessary to obtain it in a biochemically pure form, separated from other known growth factors produced by epidermal cells. Moreover, this step was necessary to ensure reproducibility and potential commercial availability of the factor(s), as only molecules purified to homogeneity can be analyzed by sequencing and prepared in large quantities by molecular cloning techniques or synthesis.

Although many growth factors have been purified to date, each purification represented a different task, based on the unique properties of the growth factor to be isolated. However, in all instances it was proven, that cells or tissues contained high biological activity, but small quantities of growth factors. Many growth factors, in order to be purified to homogeneity had to be isolated from as much as 20 kg of tissue material. Thus, the initial step toward the purification of EDF(s) was to analyze different

tissues or cultured cells for the presence of EDF and quantitate the amounts of EDF present for identification of the potentially richest source. The second prerequisite for successful purification was an easy, reproducible and reliable bioassay.

Purification and Biochemical Characterization of EDF(s)

A) Bioassay

Two bioassays were originally considered and tested. The first one utilized a melanoma cell line (M₄) derived from a primary tumor that responded to the addition of epidermal cell supernatants or extracts by DNA synthesis. This cell line was selected from a battery of cell lines originally tested. The assay was used in the first months of our studies, since we found that M₄ melanoma cells responded only to epidermal cell derived materials, but not to fibroblast derived material or a variety of other growth factors (i.e. EGF, PDGF, bFGF, TGF β). However, when purification of EDF was initiated, DNA synthesis in M₄ was induced by small m.w. fractions obtained from gel filtration columns. They corresponded in size to amino acids, presumably derived from concentrated tissue culture medium. Consequently, false positive results were occasionally obtained. These findings led to a decision for change to an assay which was much more cumbersome, but reflected more closely the biological effect expected of EDF. This assay was the collagen contraction by fibroblasts, based on the findings originally described by Bell and his collaborators (2). They found that fibroblasts when seeded into a collagen gel can cause shrinkage of collagen. This shrinkage was directly proportional to number of fibroblasts seeded and indirectly to the concentration of collagen in the gel. It was shown later by Ehrlich (3) that the degree of collagen shrinkage depends also on the type of collagen used. Type I collagen shrinks less than type III. According to current views, capability to contract collagen by fibroblasts in vitro reflects the process of wound closure in vivo. The intracellular apparatus involved in fibroblast contractility includes actin a cellular microfilament involved also in fibroblast mobility. Since it is our aim to be able to regulate wound contraction by regulating the migration/proliferation of fibroblasts, this in vitro assay appears the best available system to search for a molecule that may have such regulatory capacity in vivo. As detailed in our quarterly reports, it took much effort to standardize the assay

and make it accessible for testing of large numbers of samples. We succeeded not only in excellent reproducibility and capability to use it for quantitation, but also in minimizing the amount of material used in such studies. The assay required rat tail collagen, freshly prepared. At first the collagen was purchased from Tissue Diagnostics Inc., Bangor, PA, but we found it too costly and occasionally contaminated. Therefore a source of rat tails was secured, a procedure for preparation of collagen was developed and fresh batches of collagen were prepared every week. Details concerning preparation of collagen and performance of the assay are in the enclosed manuscript (Encl 2).

B) Source of EDF

1. Supernatant fluids from epidermal cells as a source of EDF.

Initially, as a starting material for our studies we considered using supernatant fluids of cultured epidermal cells or cell extracts prepared from human epidermal cells grown in tissue culture. We reasoned that cell supernatants will offer a source is relatively easy to purify, as it can be collected from cells grown in serum free medium. Such supernatant fluids after low speed centrifugation, to remove floating cells and cellular debris, should contain only cell metabolites and secreted factors. Supernatant fluids from normal human and pig keratinocytes grown to different degrees of confluency were tested. Similarly, we examined different keratinocyte cell lines, established by immortalization of keratinocytes using oncogenes. Only primary keratinocytes were found to secrete EDF activity. Therefore a major effort was put into producing large quantities of supernatant fluids from cultured keratinocytes. For this purpose skin was obtained (purchased) from N.Y. Hospital skin bank or from Memorial Hospital. More than 20 liters of supernatant fluid were collected and stored in deep freezer for purification of the factor. In addition keratinocytes were grown and used for preparation of cell extracts. Both supernatant fluids and cell extracts were used in our experiments investigating the most suitable method for concentration of EDF.

2. Attempts to concentrate EDF from supernatant fluids.

Titration of the EDF activity, using the above bioassay, revealed that the activity

was lost when supernatants were diluted at 1:8, or higher, suggesting low amounts of EDF to be present in culture medium. To proceed with the purification, it became obligatory to concentrate EDF activity. Of different modes of concentration, we considered at first Amicon ultrafiltration, as it was used for concentration of other growth factors. Moreover, new membranes became available noted for improvements in retaining growth factors with minimal losses. Membranes of different porosity were used, and found that only membranes with 500 m.w. cut off retained the activity. Thus, use of Amicon ultrafiltration has proven to be impractical, as it took too long to concentrate the material. Concentration by lyophilisation was successful, however, only to a limit as salt concentration increased when the material was concentrated. It was difficult to dissolve the material in a small volume and impossible to test in the bioassay unless diluted to the original volume. When dialyzed, losses that occurred were too high to consider this approach feasible. Many different approaches, such as the use of volatile buffers, ammonium sulphate precipitation, use of desalting columns following lyophilisation, have all been attempted resulting only in marginal success. From a variety of materials tested for their capacity to bind the factor, Q-Sepharose (an ion exchange column) was found to be the most suitable. For concentration of supernatant fluid containing EDF it has, however also proven to be impractical as we found that phenol red (indicator of pH, present in tissue culture medium) bound to Q-Sepharose with high affinity, possibly displacing the binding of the factor to the column. Experiments were performed in an attempt to remove phenol red from the supernatant fluid by Q-Sepharose followed by dilution of the material eluted and subsequent binding of the factor. The level of concentration was found to be unsatisfactory, particularly when larger amounts (100 ml to 1 liter) of supernatant fluids were applied. Different sizes of columns and different volumes of starting materials were applied with no positive results. Since basically all attempts to concentrate EDF from cell supernatants failed, we turned to cell extracts.

3. Epidermal cell extracts as a source of EDF.

There were considerable disadvantages to using epidermal cell extracts as a source for purification of EDF, mainly that the number of unrelated proteins was much

greater. Consequently it meant that in the process of purification additional steps will have to be added to achieve homogeneity of the isolated material. An additional concern was the presence of proteases in cell extracts that may cleave the factor in a non-physiological mode, resulting in the loss of biological activity. The third concern was that EDF may be naturally cleaved prior to secretion and that the forms isolated from cell extracts may be larger than the final product obtained in the supernatants. To clarify some of these concerns preliminary experiments were designed to compare the biological properties of cell extracts with those already established for EDF derived from epidermal cell supernatants. As expected, the amount of total protein in cell extracts was approximately 200 fold higher than in the supernatants. However, the biological activity was 400-800 x greater in cell extracts than in the supernatant fluids. (These values were calculated from titration experiments of the starting materials, using the collagen contraction assay.) (see Fig. 1) Thus, cell extract contained 2-4 x more biological activity per microgram of protein, and offered a 200 fold concentrated starting material, compared to the cell supernatants. Comparison of biochemical characteristics, i.e. alkaline and acid stability, heat stability etc. (see enclosed table I) did not reveal differences in the behaviour of EDF(s) from these two sources, suggesting that the enzymatic activity present in cell extracts did not interfere with the biological activity of EDF(s). Moreover, addition of enzyme inhibitors to cell extracts did not reveal any improvements in retaining the biological activity. We thus concluded that the stability of EDF in cell extracts is the same as in the supernatants. As preparation of cell extracts from cultured human epidermal cells was very expensive and extremely laborious a decision to use pig skin was made based on the assumption that EDF, similarly as other growth factors, is highly conserved in evolution. It seemed feasible to project that once amino acid sequence of pig EDF is established, molecular cloning can be applied to obtain the human EDF homologue.

4. Pig skin as the source of EDF.

When epidermal cell extract was prepared from epidermal cells isolated from intact skin of healthy animals, the titer of EDF activity was 4-8x lower than in cells grown in tissue culture. This finding was very interesting, suggesting that EDF is

produced in larger quantities in regenerating or rapidly growing epidermal cells rather than in quiescent cells (see Fig. 1). Since growth of epidermal cells in tissue culture resembles the process of epidermal regeneration, following wounding in vivo, we decided to perform superficial wounding of the epidermis in pigs, and quantitate the amount of EDF produced by epidermal cells at different times after wounding. Different modes of wounding were attempted, leading to the development of a protocol for preparation of starting material. It consisted of superficial shaving by Dermatome, set at 6-8/1000 of an inch. Testing of regenerated epidermis showed that maximal amount of starting material and highest EDF activity was obtained one week post wounding. Animals varying in age from 4-12 months were used. Comparative studies revealed that the age of animals did not influence the biological activity of epidermal cell extracts significantly. However, the size of the wounded area was found to be of importance. Animals that were wounded extensively did not heal properly. Impaired healing resulted in poor quality of epidermis and lesser amount of EDF. Based on preliminary experiments a decision was made to wound the animals no more than by two 5 cm wide shavings along the rib cage. One week later, split thickness skin (12/1000 inch) was collected, using a Brown dermatome. For preparation of cell extracts the epidermis was separated from the dermis by trypsinization. Action of trypsin was stopped by serum, the cells washed extensively to remove serum, and further processed as described in Encl. 2. To prepare sufficient amounts of starting material, we wounded 2 pigs each week and shaved the previously wounded area a week later.

C) Purification of EDF by gel filtration column chromatography. Sephacryl SF - 300

From different gel filtration columns, Sephacryl SF - 300 was found to be the most suitable to separate EDF activity into different components, based on their molecular weight. Different amounts of material were loaded onto a column of 2.5 cm x 95cm in size. The best results were achieved, when 10 ml of epidermal cell extract (diluted 1:2.5) was applied to Sephacryl SF - 300 column. The column was equilibrated in 20 mM NaPO_4 , 0.15 M NaCl at pH 8.0 and 5 ml fractions were collected at a flow rate of 4 ml/hr. The absorbance was measured at 280 nm. The column was calibrated using Blue dextran (2,000,000), Aldolase (158,000), Bovine albumin (68,000)

chymotrypsinogen A (25,000) and Phenol red (376). Two hundred microliters of 3 consecutive fractions were pooled and tested for collagen contraction inhibitory activity. We found 3 major peaks of biological activity (see Fig. 2). Their molecular weights were ~ 30,000, ~5,000, and ~ 1,000. These experiments were repeated several times with reproducible results. Of these three choices we proceeded with the purification of the lowest molecular weight component (~ 1,000) at first. All other fractions were frozen at -80°C for later use.

D) Purification of EDF by Q-Sepharose

Of different ion exchange columns, only Q-sepharose was repeatedly shown to bind EDF(s) at pH 8.0 or pH 9.0. A prerequisite for binding, we found, was low salt concentration. Therefore, positive fractions eluted from Sephacryl SF - 300, at the tail end of the column (m.w. ~ 1,000), were pooled and diluted with 20 mM TRIS, pH 8.0 to 0.03 M final concentration of sodium chloride. The material was loaded onto a Q-sepharose (Pharmacia) column (1 cm x 5 cm) equilibrated with 20 mM TRIS, 0.03 M NaCl at pH 8.0. The column was washed with the same buffer and eluted with 0.1 - 0.7 M NaCl gradient. Aliquots of each 2 ml fraction adjusted to 0.15 M NaCl concentration were tested. Since the bioassay was testing for inhibitory activity, extreme attention was given to proper controls. Each test included: the starting material, the buffers used, salt solutions, the standard epidermal cell extract, and the standard in the presence of different buffers used, in addition to the tested samples. As shown in the enclosed graph (Fig. 2), the biological activity was detected in tested samples eluting from Q-Sepharose between 0.35 - 0.45 M NaCl concentration.

Purification of Low Molecular Weight EDF

A. Gel Filtration Chromatography

As described above, separation of EDF activity was achieved by gel filtration columns SF-300. Six conventional gel filtration columns were done. All fractions were evaluated for their protein content by spectrophotometry and absorbance read at 280 nm. The biological activity was tested using collagen contraction inhibition assay. As previously, three major peaks of biological activity were detected. However, variations

in the distribution of biological activity in the described three peaks were observed. Similar biological activity was detected in all 6 columns at the position of 30 kd EDF, but the biological activity in peaks at ~ 5 and ~ 1 kd varied. Thus, we considered that the small m.w. components may be proteolytic degradation products of the 30 kd protein. To investigate this possibility, protease inhibitors were added to the starting material. At first we investigated whether protease inhibitors, ie. Phenylmethyl sulfonyl fluoride (PMSF) or Benzamidine affect the collagen contraction assay and/or the activity of EDF. As shown in the enclosed Table 2 and Fig. 4, neither PMSF nor benzamidine influenced EDF activity in the biological assay. Moreover, even in the presence of protease inhibitors both low molecular weight peaks were found to be present, when subjected to gel filtration chromatography. These experiments suggested that the low molecular weight components are not proteolytic degradation products of EDF 30.

B) Ion Exchange Chromatography

Low molecular weight fractions from two SF-300 gel filtration columns, with significant collagen inhibitory activity were pooled. The pooled material (80 ml) was diluted 5 fold (to reduce salt concentration to 0.03 M) with 20 mM, pH 8.0 TRIS and applied to a Q-Sepharose column. The column was eluted with 0.5 M NaCl.

Biologically active fractions eluted from Q-Sepharose (4 ml) were diluted again (to final salt concentration of 0.03 M) and applied to an FPLC-Mono Q column (Pharmacia). Elution of the bound material was achieved with a multilinear gradient 0.05 - 2 M NaCl in 20 mM TRIS, pH 8.0. The biological activity was detected in fractions eluted with 0.6 - 0.9 M NaCl (see Fig. 5A). Fractions displaying biological activity had no detectable protein content by optical density measurements at 280 nm. They were pooled into a 6 ml batch.

C) SDS Gel Electrophoresis

One (1) ml of the above pool was precipitated by ethyl alcohol and applied to a 15% SDS polyacrilamide gel electrophoresis. When stained with silver stain a faint band around 30 kd and even fainter band at 10 - 15 kd was detectable. As expected the low molecular weight component was not visible in the gel. Based on the results of gel

electrophoresis it was obvious that the material purified by the three previous steps (gel filtration, Q-Sepharose and Mono Q) was still heterogeneous and needed to be subjected to further purification steps.

D) Reversed Phase Chromatography

Prior to considering to subject EDF to reversed phase chromatography, buffers that needed to be used in such a column were tested in the collagen contraction inhibition assay. As can be seen from the enclosed Fig. 4, we tested ammonium acetate (10 mM), ethyl alcohol (5%) and isopropanol (5%). The enclosed results show that the buffers used did not affect collagen contraction by fibroblasts. Interestingly, the inhibitory activity of EDF was increased in the presence of ethyl alcohol or isopropanol. The reason for this increase in the activity is not understood at the present time.

Three and one half (3.5) ml of partially purified EDF (from the total 6 ml) obtained from HPLC Mono Q column was sent to AMGEN. It was applied to a reversed phase C₄ column and eluted with 2 buffers. Buffer A: 25 M ammonium acetate pH 6.0 and buffer B: 25 M ammonium acetate, pH 6.0 in isopropanol. 25 fractions were collected and dried. They were resuspended and tested by us in collagen contraction inhibition assay. Biological activity was detected in two fractions eluted in the beginning (see Fig. 6), suggesting that EDF did not bind to the column. The total volume of the two fractions was 4.2 ml and the titer was 1/16. The result obtained suggested that the conditions for proper separation had to be improved and therefore we began to prepare a new batch of material for reversed phase column chromatography.

Purification of High Molecular Weight EDF

We also examined the behavior of the high molecular weight EDF. When applied to both, Q-Sepharose and Mono Q ion exchange columns the elution profile appeared to be similar to the low mw EDF (for comparison, see Fig. 5A and 5B).

The material eluted from Mono Q column was applied to a Con A column. EDF activity did not bind to the column, suggesting lack of glycosylation sites.

Comparative Studies of EDF and Fibronectin in Collagen Inhibition Assay

This study was initiated for two reasons: 1) to establish the possible role of fibronectin in promoting or inhibiting fibroblast induced contraction of collagen and 2) to exclude the possibility that EDF activity observed in the assay may be due to fibronectin.

As shown in Table 3 fibronectin at 0.1, 1 and 10 μg was added to the standard collagen contraction assay. Addition of 0.1 and 1 μg caused no change in fibroblast induced contraction of collagen gels, compared to the controls without fibronectin. 10 μg of fibronectin, however, inhibited collagen contraction. When the same amounts of fibronectin were added to the assay in the presence of an epidermal cell extract diluted at 1:400, only 10 μg of fibronectin had a significant inhibitory effect, compared to the EDF control.

Since we know that EDF in the collagen contraction assays works at nanogram quantities, while for fibronectin 100 - 1000 fold larger amounts were needed to observe an effect, the possibility that EDF's effect would be due to fibronectin was for the time being excluded. More experiments will have to be done, however, in the future to clarify this question.

Effects of EDF(s) on Epidermal Grafts In Vivo

Two experiments were performed on pigs using autologous epidermal grafts grown in tissue culture and transplanted onto a full thickness wound bed. The grafts were covered with Release soaked in epidermal derived factor (EDF). The factor was applied in one instance four times, every second day, and two times in the other. Consequently, the epidermal cells replicated rapidly and covered the wounds with thick epidermis in 4-6 days. Unfortunately, the thick coverage caused retention of the wound fluid. Due to this, epidermis was found to be separated from the dermis. The experiments will be repeated with shallow incisions to be performed in the epidermal graft 48 hours after their placement on the wound bed.

II. STUDIES AIMED AT THE UNDERSTANDING OF ALLOGENEIC EPIDERMAL CELL GRAFT REJECTION

Epidermis, the outermost layer of skin, is composed of stratified squamous epithelium. Its function is to provide a physiological barrier and protection of the body. Because of its functional and cosmetic importance means for epidermal cell replacement, when lost by injury, have long been sought.

As skin has proven to be one of the organs most difficult to transplant allogeneically, findings that epidermal cells when grown in tissue culture can be freed of Langerhans cells, led to the belief that new approaches to allogeneic epidermal cell transplantation will become available. However, based on the work of many investigators, it became obvious that even in the absence of Langerhans cells, the antigen presenting cells of the epidermis, allogeneic epidermal keratinocytes were always rejected. Thus, our goal was to understand the mechanism of allogeneic epidermal cell graft rejection.

Induction of Class II Major Histocompatibility Antigens (MHC) on Keratinocytes by Allogeneic Dermis

It was reported previously (4,5,6), that in the skin transplanted allogeneically, early in the process of rejection, epidermal cells were induced to expression of class II major histocompatibility antigens, Ia antigens. These antigens, found to be constitutively expressed on Langerhans cells of the epidermis, and are known to be necessary for antigen presentation to T-lymphocytes, cells responsible for graft rejection. It was presumed that Ia induction on epidermal cells was secondary, caused by gamma interferon, produced by lymphocytes, present in rejecting grafts. This immune interferon has been shown by many, to be able to induce Ia expression not only on keratinocytes, but also on fibroblasts. To investigate whether the mechanism of class II induction on keratinocytes is due to exogenous gamma interferon, we established an in vitro system. The system was described in detail in the summary report of our previous contract.

A) In Vitro Ia Induction Assay

Briefly, human or pig keratinocytes were grown in tissue culture until multilayer

epidermal structures were formed. They were removed from the tissue culture vessels as sheets, using the enzyme Dispase. A split thickness graft of reticular dermis was obtained from pigs, using a Brown dermatome. Following thorough wash of the dermis, epidermal graft was "transplanted" on the dermis and the composite graft placed onto a stainless steel grid in a Petri dish filled with tissue culture medium.

In experiments with human keratinocytes grown in vitro for 3 weeks and placed on pig dermis, induction on Ia antigen was observed in the following fashion: on **Day 3** - all cells were Ia⁻ (negative); on **Day 5** - 20% of keratinocytes were Ia⁺ (positive); subsequent increase to 40% was found on **Day 7** and **Day 9**. **Day 11** - the number of positive cells reached 90%. This gradual increase was followed by a decrease of Ia⁺ cells on **Days 13** and **18** to 15%. Human fibroblasts exposed to the dermis of the same donor as were keratinocytes in the above experiments, were not induced to Ia expression. Negative results obtained with fibroblasts suggested that the induction of class II histocompatibility antigens seen with keratinocytes was not due to the presence of exogeneous interferons in the dermis, but that if interferons are the cause they may be autocrine in nature. To confirm an already well accepted finding that gamma-interferon can be an inducer of Ia expression in fibroblasts, and that this finding applies to the fibroblasts used in the above studies, we tested recombinant gamma-interferon on the above fibroblasts and showed induction of Ia antigens with 100 U/ml of gamma-interferon. Thus, we provided further evidence that induction of class II major histocompatibility antigens on epidermal keratinocytes, when exposed to allogeneic dermis was not due to exogeneous gamma interferon.

B) Neutralization Assay

To confirm and clarify our preliminary evidence that expression of Ia antigens on keratinocytes may be due to induction of an autocrine gamma-interferon, neutralization assays with anti-gamma interferon were performed. The assay system used was modified to use less media, therefore to require less of costly antibodies. Smaller dishes were custom made for us by the mechanical shops of SKI. These dishes needed only 10 ml media/each, compared to 50 ml used previously. The experiments were set as previously described using fresh pig dermis and human keratinocytes

grown in tissue culture. Anti human gamma-interferon antibodies at 1 U/ml were added at the time of co-cultivation and every second day the medium was exchanged for the same. As shown on the enclosed graph (Fig. 7), 90% induction of Ia antigen was observed on day 7 in the control samples. In contrast, in the presence of antibodies to gamma interferon a significant suppression of Ia induction was found (15% positive cells). In another assay (not shown) a complete suppression of Ia-antigen was seen in the presence of anti-gamma interferon antibodies. In this case, however, controls (in the absence of antibodies) showed 25% maximal induction only.

C) Hybridization In Situ

In Addition to the above experiments, we also succeeded in obtaining good quality in situ hybridization data. They represent repeated, previously described experiments using a gamma interferon probe to localize messenger RNA in the epidermal cells cultured on the pig dermis. The data are now sufficient to support our findings that allogeneic or xenogeneic dermis can induce keratinocytes to gamma-interferon production and expression of class II antigens. Manuscript for publication is being prepared.

D) Induction of Class II MHC Antigens on Keratinocytes by "Treated" Dermis

It was reported previously (7) that frozen dermal allografts supported the engraftment and expansion of autologous epidermis. Naturally, it became of interest to examine whether frozen dermis retains its capacity to induce Ia on cultured keratinocytes. We therefore froze (pig) dermis, using the protocol previously described, and used it in in vitro "transplantation" experiments. Frozen dermis induced Ia expression in keratinocytes after 14-16 days of co-cultivation in vitro. The induction was however only 5% on days 14 and 16 and 10% on day 20, compared to the control, non-frozen dermis in which 40-90% of the epidermal cells were induced to express Ia antigens in 7-11 days. Repeated experiments confirmed our findings. In all instances we found that freezing significantly suppressed the dermal activity responsible for Ia induction in keratinocytes. However, repeated freeze - thawing (3X) did not have additional effects. For example: Controls were induced on day 5, reached maximum

on day 9 (60% positive cells) and declined gradually to day 20. In contrast, 1x frozen-defrosted dermis induced expression of Ia only after 12 days of cocultivation, and only in less than 10% of the cells. Three (3) times frozen dermis induced Ia expression after 14 days also on less than 10% of the cells tested. To eliminate the possibility that DMSO, used in the freezing procedure, might have interfered with the induction, a control experiment for the effect of DMSO was performed. It was found that when dermis was exposed to the same concentration of DMSO as used for freezing, subsequently washed but not frozen, induction of Ia by keratinocytes was not affected.

We also examined the effects of prolonged exposure of dermis to proteolytic enzymes such as trypsin (at 0.25%, 12 hrs at 4°C, concentration known not to be harmful to cells) on the Ia induction. We found that trypsinized dermis acted similarly as frozen dermis; Ia induction occurred later than in the controls (on day 12), but the numbers of positive cells were slightly higher than seen with frozen dermis (20-30%). A combination of trypsinization and freezing, however, completely abolished the activity. These findings may be important for possible clinical use of dermis in full thickness (third degree) burn wounds.

E) Induction of Class II MHC in "Conditioned" Epidermal Cells by Allogeneic Dermis

In addition we performed experiments using pig epidermal cells, "conditioned" with allogeneic dermal extracts, while grown in tissue culture and control cells "not conditioned". Both were subsequently "transplanted" in vitro onto allogeneic dermis from the same donor. These experiments were to assess whether "treatment" of cells with dermal extracts inhibits or delays Ia-induction by keratinocytes. Limited number of experiments of this nature were done, suggesting that Ia induction is delayed but not inhibited when epidermal cells are pre-treated. Thus, treatment of dermis appears to be a more effective way to influence Ia induction in keratinocytes than "treatment" of epidermis.

F) Induction of Ia Antigens on Human Keratinocytes by Tumor Necrosis Factor (TNF) or Combination of TNF and Interferons

We investigated whether TNF, known for its capacity to induce class II antigens on

different types of cells including endothelial cells, can alone or in combination with small doses of gamma-interferon cause induction of Ia on keratinocytes. TNF at 50, 500, and 5,000 U/ml; gamma-interferon at 1, 10, 100, and 200 U/ml; and alpha-interferon at 100, 200, and 1,000 U/ml was tested on human keratinocyte cultures. In addition, a combination of TNF (5,000 U/ml) and gamma-interferon (100 U/ml) as well as TNF (5,000 U/ml) with gamma-interferon (200 U/ml) were tested for their capacity to induce Ia in keratinocytes. Gamma-interferon at 100 and 200 U/ml induced Ia in 75% of keratinocytes. TNF at 5,000 U also appeared to induce Ia in keratinocytes. Alpha-interferon gave negative results. The finding that TNF might induce Ia in keratinocytes might be very interesting, but must be repeated. We also plan to test IL₆ at the same time. These experiments should provide a solid baseline study of inducibility of Ia antigens on human keratinocytes and lead to experiments deciphering the importance of individual factors in Ia expression and may be allograft rejection.

Clinical Examination of Autologous Frozen Dermis in Full Thickness Wounds

To evaluate the potential for clinical use of frozen dermis or frozen dermis covered by autologous epidermal cells grown in vitro we prepared a series of full thickness wound beds and transplanted: 1) dermis that was frozen-defrosted 1x; 2) dermis frozen-defrosted 3x; 3) frozen-thawed dermis co-cultured with autologous epidermal cell. In addition: 4) we covered one wound with split thickness fresh autograft and 5) with sheets of autologous epidermal cells grown in tissue culture and 6) a control wound was covered with petrolatum gauze. Each wound was approximately 25 cm². All grafts in this experiment were autologous. Clinical examination on day 6 suggested take of dermal autografts (both 1x and 3x frozen) but there was no epidermal coverage. On the dermal frozen graft that was covered by epidermal cells in tissue culture there was a thin epidermal coverage. Split thickness autograft, as expected, was completely healed. Tissue grown epidermal autograft provided a thin epidermal coverage. The control wound was completely open. On day 9 and 13 - wounds covered by epidermal sheets were completely healed, they felt soft and pliable. They resembled those of split thickness skin. Wounds covered by 1x and 3x frozen-thawed dermis looked identical. There was no wound contraction. On day 9 they were partly covered by epidermis.

On day 13 there was full epidermal coverage. Wounds covered by in vitro co-cultured epidermis with dermis were not confluent covered by epithelium on day 9, but complete coverage was noted on day 13. Interestingly, some hair growth occurred in this wound. Controls on day 9 and 13 were contracted, but the remaining area following contraction was still denuded.

Preliminary histological examination of biopsies taken on day 6 and 13 gave interesting insight into the process of wound healing under these different conditions.

Wound beds covered by epidermal sheets grown in tissue culture were fully covered by a thick epithelium on day 6. The hyperplasia observed in these biopsies regressed by day 13. A thick layer of granulation tissue filled the wound.

Significant difference was between the dermal grafts that were covered by epidermal cells and plain dermal grafts. There was much less granulation tissue on the interface between the dermal grafts and fat (the grafts were placed on fat) when grafts with epidermal cells were used, than with dermal grafts alone. In addition, the fibroblast infiltration of the dermis was substantially less in dermis-epidermal grafts. This result is not surprising, since we have originally found that epidermis produces a factor (EDF) that suppresses fibroblast migration/proliferation in the wounds.

It is natural that we are now planning to extend these experiments to the use of allogeneic dermis. We plan to use frozen dermis as well as frozen-trypsinized dermis for further studies.

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FIGURE 1

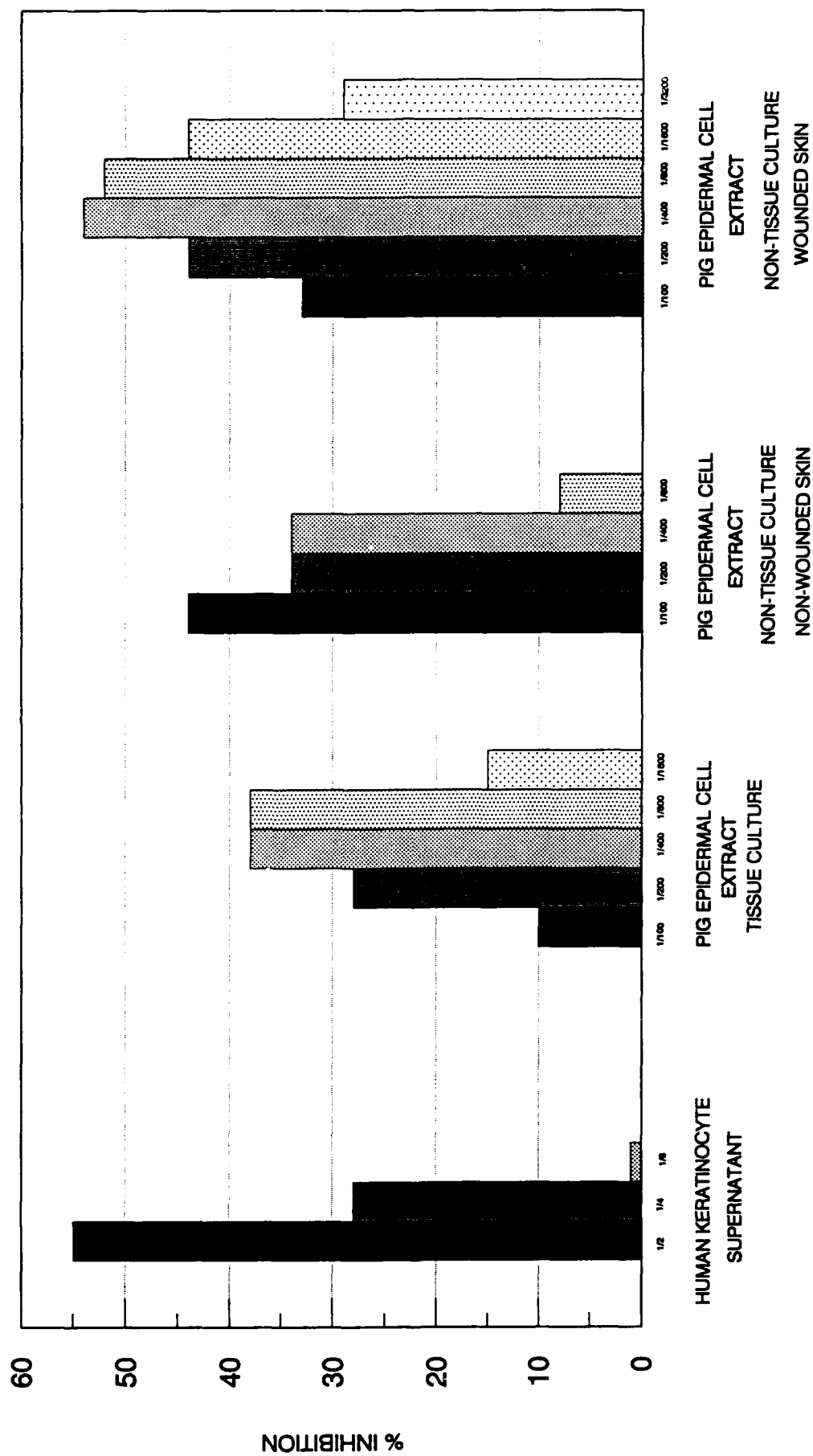


FIGURE 2

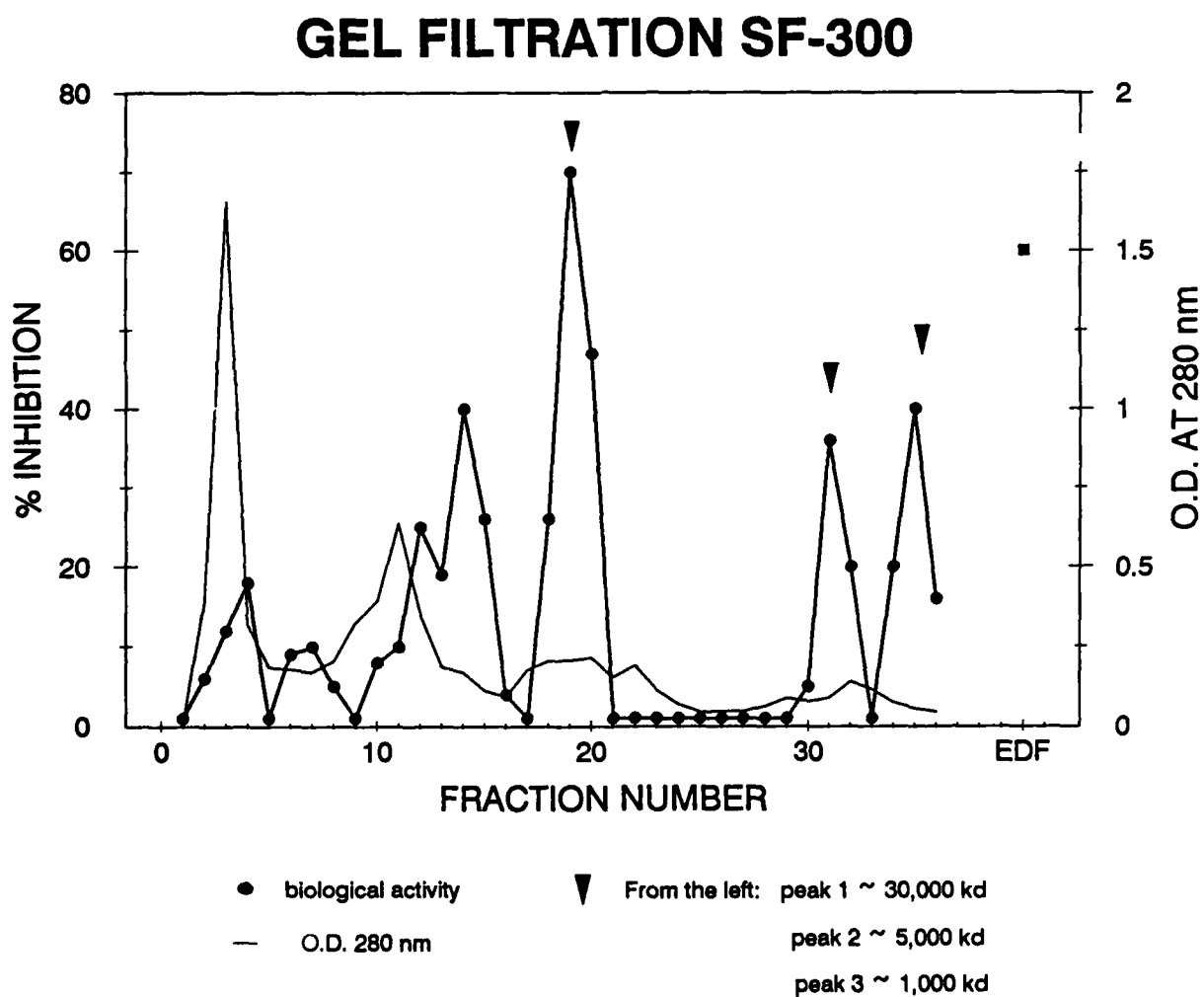


FIGURE 3

Q SEPHAROSE LOW MOLECULAR WEIGHT

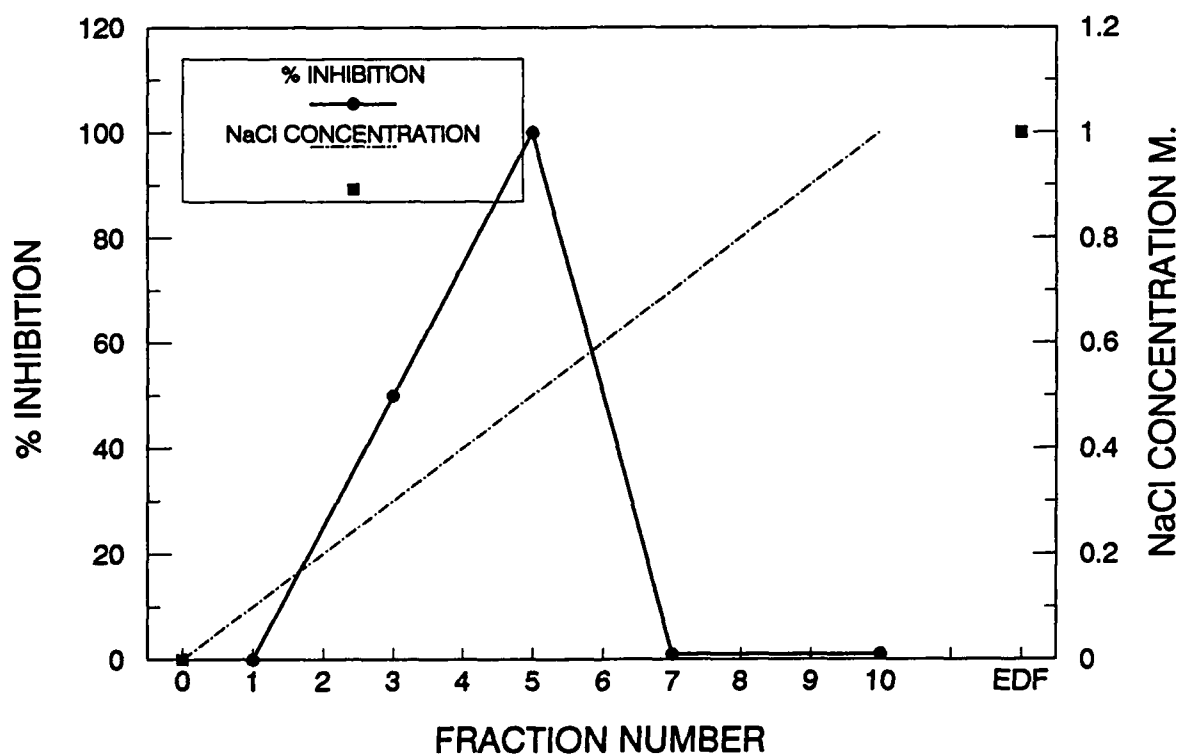
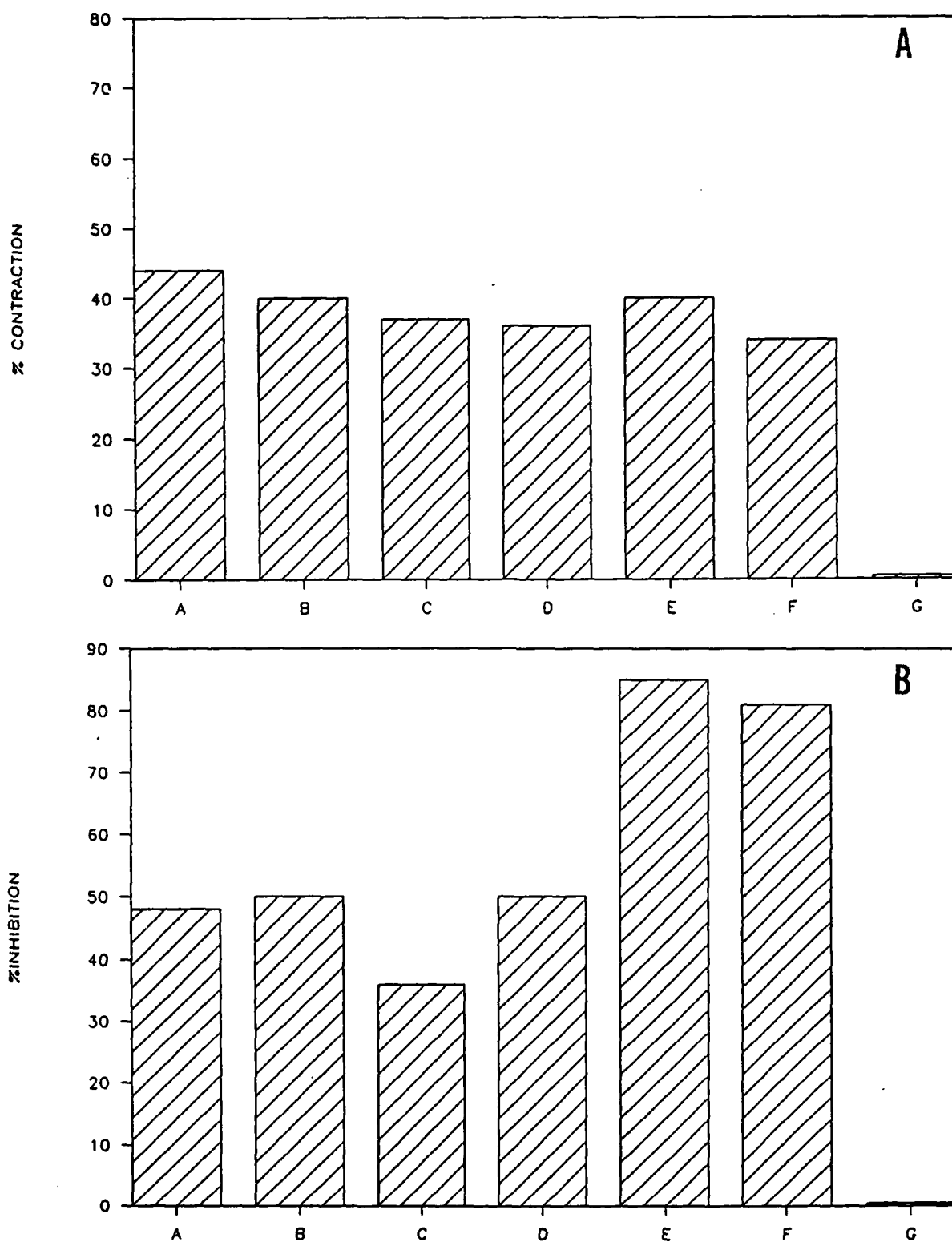


FIGURE 4

EFFECTS OF DIFFERENT SUBSTANCES ON COLLAGEN GEL CONTRACTION BY FIBROBLASTS



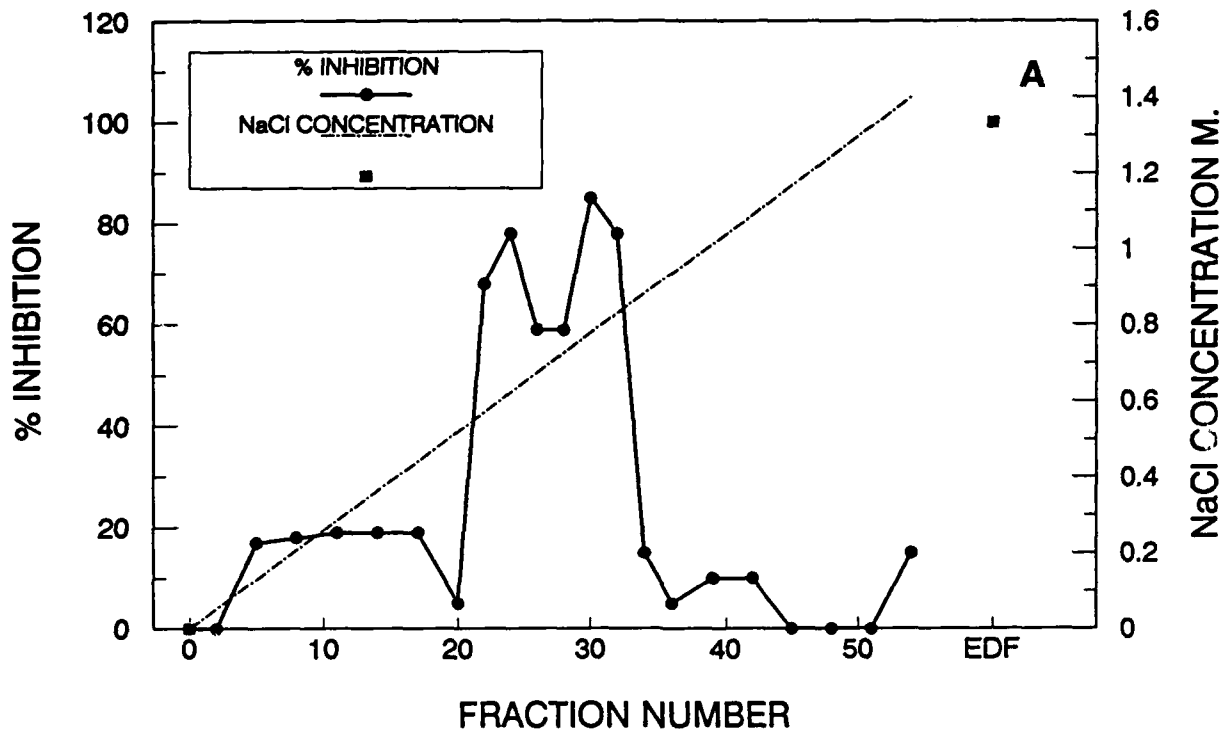
A: MEM Control B: 10 mM Ammonium Acetate (1:2) C: 0.1% Sodium Cholate (1:10) D: 10^{-5} M PMSF (1:2)
E: 5% Isopropanol (1:2) F: 5% Ethanol (1:2) G: 0.1% 2-Mercaptoethanol (1:40)

Graph A: compared to the control (A) substances B-F did not affect fibroblast induced collagen contraction at the dilutions tested. G showed toxic effect.

Graph B: % Inhibition by EDF was not affected by substances B-D. There was a significant increase in the EDF activity by E-F. G had a toxic effect.

FIGURE 5

MONO Q LOW MOLECULAR WEIGHT



MONO Q HIGH MOLECULAR WEIGHT

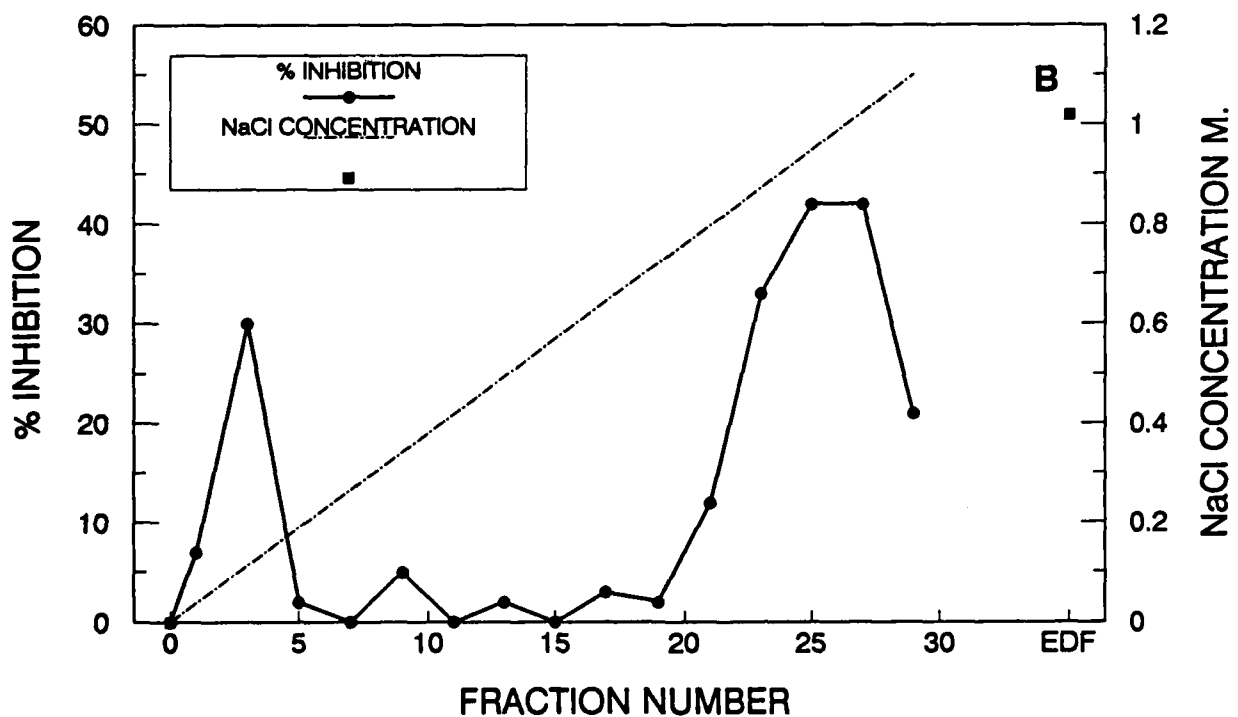


FIGURE 6

REVERSED PHASE I

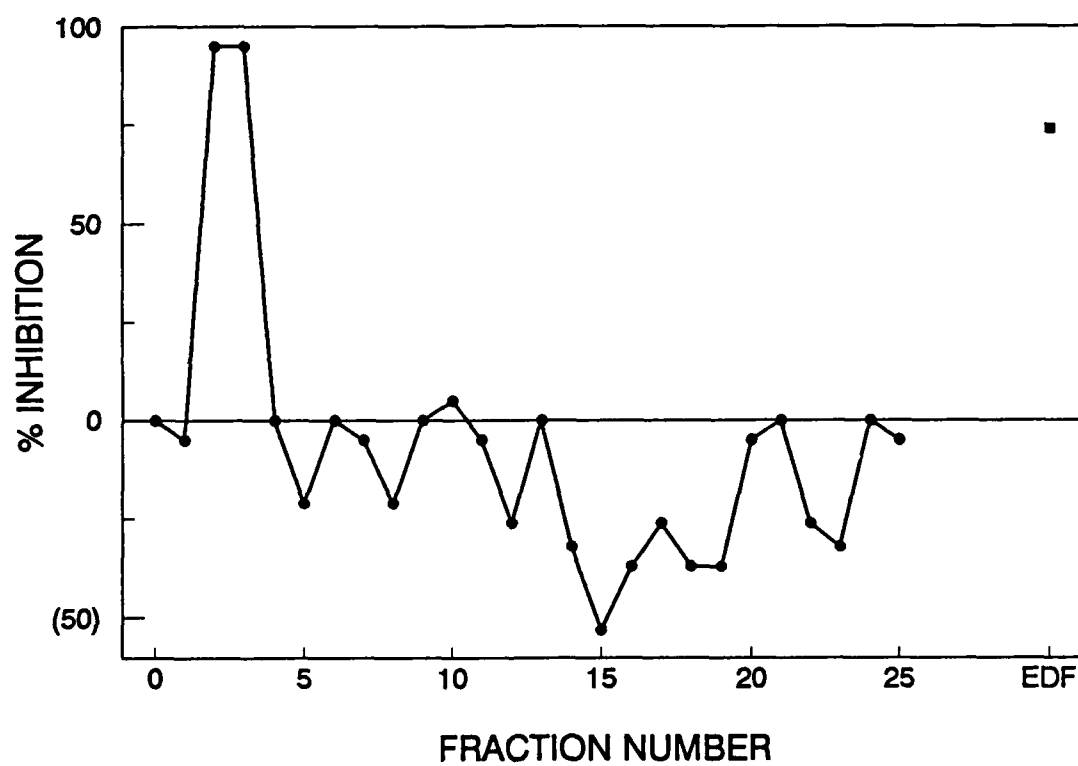


FIGURE 7

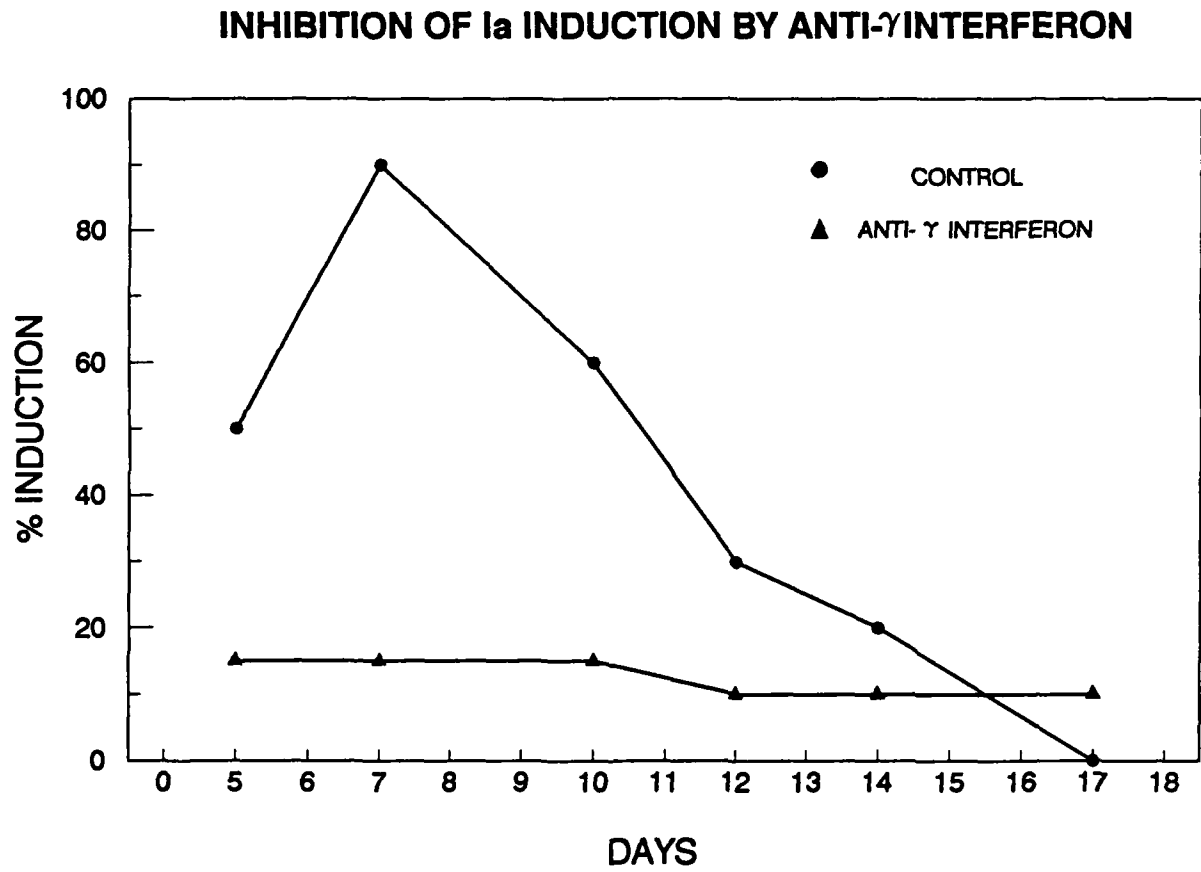


TABLE I

BIOCHEMICAL CHARACTERISTICS OF EDF

| <u>TREATMENT</u> | <u>STABILITY</u> |
|----------------------|-----------------------|
| Alkaline pH 10.0 | Stable |
| Acid Ph 2.0 | Unstable |
| Acid Ph 4.0 | Unstable |
| Heat 60°C 30 min. | Stable |
| Heat 100°C 10 min. | Unstable |
| -80°C 1 month | Stable |
| -20°C 1 month | Stable |
| 4°C 1 week | Unstable |
| Room Temp. 24 hrs. | Stable |
| Lyophilization | Stable |
| Acid Ethanol | Unstable |
| Chloroform Methanol | Unextractable |
| Acetone | Unextractable |
| 90% Ammonium Sulfate | Precipitates activity |

AMICON FILTRATION

| <u>Membrane</u> | <u>MWCO</u> | <u>Activity</u> |
|-----------------|-------------|-----------------------------|
| YM10 | 10,000 | not retained in concentrate |
| YM5 | 5,000 | not retained in concentrate |

DIALYSIS

| <u>Tubing MWCO</u> | <u>Activity</u> |
|--------------------|---------------------------------|
| 6-8,000 | activity not detected in tubing |
| 3,500 | activity not detected in tubing |
| 2,000 | activity not detected in tubing |

TABLE I

AFFINITY COLUMNS

| <u>Columns</u> | <u>Binding</u> |
|-------------------------|----------------|
| Con A | - |
| Heparin | - |
| Hydroxylapatite | - |
| Blue Sepharose | - |
| Phenyl Sepharose | - |
| Lysine Sepharose | - |
| Octyl Sepharose | - |
| Remazol Blue Agarose | - |
| Dextran Sulfate Agarose | - |

ION EXCHANGE COLUMNS

| | |
|----------------------|------------------------------------|
| CM Sephadex pH 6.0 | does not retain activity |
| DEAE Sephacel pH 8.0 | weakly retains activity |
| Q Sepharose pH8.0 | retains activity (elute 0.3M NaCl) |

TABLE II**THE EFFECTS OF BENZAMIDINE ON COLLAGEN
CONTRACTION BY FIBROBLASTS**

| SUBSTANCES TESTED | DILUTION IN THE OVERLAY | % INHIBITION | |
|-------------------|----------------------------|--------------|----|
| | | - | + |
| EDF | 1:200 | 100 | 97 |
| | 1:400 | 100 | 82 |
| | 1:800 | 55 | 60 |

- = Without Benzamidine
+ = With Benzamidine

TABLE III**THE EFFECTS OF FIBRONECTIN ON FIBROBLAST
INDUCED COLLAGEN CONTRACTION**

| FIBRONECTIN (g) | % CONTRACTION |
|------------------|---------------|
| 0.0 | 45 |
| 0.1 | 44 |
| 1.0 | 44 |
| EDF (1:400) | 32 |

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Chronic Wounds

**EPIDERMAL CELL DERIVED FACTOR (EDF): PARTIAL
PURIFICATION AND CHARACTERIZATION**

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INTRODUCTION

Since the initial discovery of epidermal growth factor (EGF) and nerve growth factor (NGF) (Levi-Montalcini, Cohen, 1960; Cohen, 1962), large numbers of polypeptide growth factors have been isolated from different tissues (Burgess, 1988). They were shown to operate according to the same principles governing classic hormones and their receptors, and act in an autocrine or paracrine fashion. A variety of growth factors and lymphokines have also been shown to be produced by epidermal cells (In Milestone and Edelson, 1988). Of these EGF, transforming growth factor-alpha (TGF-alpha), basic fibroblast growth factor (b-FGF) and interleukin-1-alpha (IL-1-alpha) have been implicated in the stimulation of epidermal cell regeneration. In addition, potent inhibitors of epidermal cell proliferation (chalcones) have been previously identified in epidermal cells (Reichelt et al, 1987). The effects of most known and well characterized growth factors on fibroblasts have been shown to be stimulatory *in vivo*, resulting in an increase in fibroblast cell numbers and generation of granulation tissue.

We have shown previously, that epidermal cell extracts and supernatant fluids of cultured epidermal cells stimulated keratinocytes but inhibi-

ted fibroblasts. This biological activity was ascribed to an epidermal cell derived factor(s) (EDF) (Eisinger et al, 1988a, 1988b). The in vitro effect of EDF on epidermal cells resulted in an increased number of rapidly proliferating colonies composed mainly of basal keratinocytes. In fibroblast cultures EDF inhibited the ability of fibroblasts to cause contraction of collagen sponges. Epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF), platelet derived growth factor (PDGF), transforming growth factor β (TGF- β), nerve growth factor (NGF), and extracts of WI-38 cells (human embryonic lung fibroblasts) did not have this inhibitory activity. Application of EDF to surgical wounds stimulated extensive migration and proliferation of keratinocytes from remnants of glands, hair follicles and wound edges. The restoration of complete epidermal coverage occurred twice as rapidly as that of the control wounds. In addition, regenerating dermis in the EDF treated wounds contained 1/5 to 1/15 as many cells as cells treated with EGF, urogastrone, TGF- β or phosphate buffered saline.

These findings implied a possible role for EDF(s) in both positive and negative feedback mechanisms and the maintenance of homeostasis in the epidermis and dermis. In view of their importance for understanding the normal mechanism of wound healing and potential for controlling abnormal or clinically undesirable manifestations, i.e. scar formation, we attempted to biochemically purify and characterize the EDF molecules. This paper describes partial purification and characterization of EDF(s) derived from pig skin.

MATERIAL AND METHODS

Preparation of Epidermal Cell Extracts

Pig epidermal cells were grown in vitro as previously described (Eisinger, 1985). Confluent

cultures (grown for 3-6 weeks) were washed twice with Dulbecco's phosphate-buffered saline (PBS), removed by scraping with a rubber policeman and pelleted at 180 x g for 10 min. In addition pig skin, wounded 7 days prior to collection by superficial shaving with a Dermatome, was used as source of epidermal cells. Skin obtained from donor pigs was washed extensively in antibiotic mixture (penicillin, streptomycin, Fungizone), cut into strips and enzymatic separation of epidermis from dermis was achieved in a 0.25% trypsin solution (DIFCO 1:250) at 37°C for 2 hours or at 4°C overnight. Following a thorough wash in PBS the epidermis was mechanically separated from the dermis using watchmaker forceps. Epidermal cells were pelleted at 180 x g. The pellets were resuspended in an equal volume of PBS, sonicated twice for 15 seconds, and diluted 1:2.5 in PBS. The suspension was clarified by two consecutive ultracentrifugation steps at 16,000 x g for 20 min. and 150,000 x g for 45 min. The resulting clarified epidermal cell extract, considered 1:2.5 diluted starting material was divided into aliquots and frozen at -70°C.

Measurement of Fibroblast Contractility In Vitro

Human foreskin fibroblasts, HFSF-132 (kindly provided by P. Ehrlich) were grown in tissue culture in minimal essential medium with Earle's salts, containing nonessential amino acids, 2 mM L-glutamine, antibiotics and 7.5% fetal bovine serum (cMEM). Cells passaged 16-27 times were used for the experiments.

Three dimensional collagen gels were prepared by minor modification of previously described methods (Montesano, Orci, 1988; Bell et al, 1979; Buttle et al, 1983). Type I collagen was extracted by stirring adult rat tail tendons for 16 hrs at 4°C in a sterile 10 mM hydrochloric acid solution (300 ml for 1.5 g of tendon). The resulting solution was centrifuged at 25,000 x g for 50 min. at 4°C. The supernatant was then precipitated with sodium chloride at 10% final

concentration. Mixing at 4°C for 6 hrs was followed by centrifugation at 30,000 x g for 50 min. at 4°C. The pelleted precipitate was re-dissolved in 2.5 mM HCl (~ 100 ml) and stirred at 4°C for 24 hrs. Dialysis tubing (6-8,000 mwco) was used to extensively dialyze the material against 2.5 mM HCl.

For incorporation into collagen gels, cells were harvested from confluent cultures using 0.05% trypsin and 0.02% EDTA counted and adjusted to a concentration of 1×10^6 cells/ml in cMEM. They were placed on ice together with other ingredients. Each 35 mm plastic petri dish (Falcon, 1008) was filled with 0.6 ml collagen gel solution, 0.6 ml 2x MEM, 0.15 ml FCS, 0.05 ml MEM and 1×10^5 cells in 0.1 ml. They were allowed to gel for approximately 10 min at room temperature and the test material diluted in cMEM (total volume 0.5 ml) was layered on top of the gel. Twenty-four hrs later the gels were detached from the walls by gently tilting and rotating the dish and freeing the edges with a size 10 blade.

Gel contraction was quantified at 48-72 hrs after application of the test materials. The dishes were placed on a metric scale graph paper and the major and minor axes of the collagen gels were measured. The area of the gels was calculated and expressed as a percentage of the initial area. Inhibition of collagen contraction was calculated from the ratio of percent contraction of triplicate samples containing factor and control samples in the absence of factor. For the final evaluation values obtained for 100% contraction of control samples represented 0% inhibition of contraction.

For comparative studies of different EDF preparations, or fractions obtained in the process of purification, their collagen contraction inhibition activity was compared to an EDF standard. The standard was defined as 50% contraction inhibiting activity of an epidermal cell extract shown to be active in the assay to

a final dilution of 1/3200. Inhibition of contraction by a tested sample which was equal to or less than 25% of the standard was considered negative.

Gel Filtration and Ion Exchange Chromatography

Concentrated starting material, (pig epidermal cell extract), (10 ml) was applied to a Sephacryl SF-300 (Pharmacia) column (2.5 x 95 cm), equilibrated in 20 mM NaPO_4 , 0.15 M NaCl, pH 8.0 and 5 ml fractions were collected at a flow rate of 4 ml/hr. The absorbance was measured at 280 nm. The column was calibrated using Blue dextran (2,000,000) Aldolase (158,000) Bovine serum albumin (68,000) Chymotrypsinogen A (25,000) and Phenol red (376). Two hundred microliter aliquots of 3 consecutive fractions were pooled and tested for fibroblast inhibitory activity in collagen gels. Fractions which tested positive, were pooled and diluted with 20 mM TRIS, pH 9.0 to a 0.03 M final concentration of sodium chloride. The material was loaded onto a Q sepharose (Pharmacia) column (1 x 5 cm), equilibrated in 20 mM TRIS, 0.03 M NaCl at pH 9.0. The column was washed with the same buffer, and eluted with a 0.1 - 0.7 M NaCl gradient. Aliquots of each 2 ml fraction, adjusted to 0.15 M salt concentration, were tested. Biologically active fractions were pooled again, clarified by centrifugation at 100,000 x g for 1 hr, diluted with TRIS buffer to 0.03 M final salt concentration and applied to FPLC - Mono Q column (Pharmacia) equilibrated with 0.05 M NaCl in 20 mM TRIS, pH 8.0. Following application of the test material the column was washed with 0.05 M NaCl in TRIS buffer, and eluted with a multi-linear gradient of 0.05-2 M NaCl in 20 mM TRIS, pH 8.0. Aliquots from one ml fractions were tested for biological activity.

Assay of EDF Activity in Vivo

Domestic outbred swine were anesthetized with

ketamine hydrochloride, anesthesia was maintained by a mixture of halothane, nitrous oxide, and oxygen. The operation site was shaved and cleansed with betadine and 70% (vol/vol) ethyl alcohol. Wounds 0.040 inch (1 mm) deep were created on the sides of the thorax with a Brown dermatome. Nonadhesive dressing (Release, Johnson & Johnson) was cut to fit the size of the wound, soaked in the materials to be tested, and applied to the wound bed. Control wounds were treated with Release soaked in PBS. The dressing was covered with multiple layers of gauze held in place by silk ligatures and protected by an Elastoplast bandage. After surgery the pigs received analgesics such as Tylenol to alleviate discomfort. The wounds were observed at 2 to 4 day intervals and 3 mm punch biopsy specimens were taken from the center of the wound. After 5 days the wounds were redressed with Release with saline solution only. By 12-14 days after surgery wounds were usually left uncovered.

Histology

The 3 mm punch biopsy specimens were fixed in Bouin's solution overnight and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin and evaluated by light microscopy.

RESULTS

Activity of EDF(s) as measured by inhibition of fibroblast contraction of collagen gels

Collagen contraction by fibroblasts have been shown to be i) proportional to the number of cells embedded in the gel and ii) inversely related to the collagen concentration (Bell et al, 1979). We used a low concentration of collagen (1.3 mg/ml), and moderate number of fibroblasts (1×10^5 /2 ml of gel mixture). Under these conditions fibroblasts produced a slow and moderate contrac-

tion of collagen gels and EDF inhibited contraction of collagen. The % inhibition of collagen contraction was dependent on the amount of EDF applied to the assay. A linear concentration dose dependent response was however, not achieved. Consequently the assay was considered semiquantitative only.

Aided by the EDF standard preparation, comparative studies of different cell extracts were made. It was found that cell extracts prepared from cells grown in tissue culture and those actively growing in vivo after superficial wounding had similar collagen contraction inhibiting activity. On the other hand, non-wounded skin derived epidermal cell extracts had approximately 20 times less activity, suggesting a functional role for EDF(s) in the wound healing process. Since harvesting skin from pigs was much more economical than growing cells in culture, we used donor pigs as a source of starting material.

Biochemical Characteristics of EDF

| Table 1 Treatment | Stability |
|--------------------------------|----------------|
| pH 10.0 | + |
| pH 2.0, 4.0 | . |
| 100°C - 10 min. | . |
| 60°C - 30 min. | + |
| 4°C - 2 weeks | + |
| -80°C - 1 month | + |
| -20°C - 1 month | + |
| Freeze-Thaw (6x) | + |
| Room Temp. - 2 weeks | + |
| Lyophilization | + |
| Acid Ethanol | . |
| Affinity Chromatography | Binding |
| Con A | . |
| Heparin | . |
| Hydroxylapatite | +/- |
| Blue Sepharose | . |
| Phenyl Sepharose | . |
| Lysine Sepharose | . |
| Octyl Sepharose | . |
| Remazol Blue Agarose | . |
| Dextran Sulfate Agarose | . |
| Ion Exchangers | Binding |
| CM Sephadex pH 8.0 | . |
| DEAE Sephadex pH 8.0 | +/- |
| Q Sepharose pH 8.0 | + |

Table 1 lists different treatments of epidermal cell extracts and their effects on biological activity of EDF(s) as detected by the collagen contraction assay. EDF(s) have been found to be stable at alkaline pH (pH 10) without any loss of the biological activity. In contrast, all EDF activity was lost when subjected to pH 4.0. Decline in the activity was found even at pH 5.5. EDF(s) are therefore acid sensitive, a property which interfered with the use of many purification procedures that require low pH. When treated at 60°C for 30 min activity was retained, but titration of these material revealed an approximate 50% loss. All activity was lost by boiling for 10 min. The biological activity was retained at 4°C and room temperature for 2 weeks. Freeze thawing (6x) and storage at -80°C did not result in significant losses of the activity. When lyophilized all the activity was recovered following resuspension to the original volume in distilled water. To evaluate whether EDF activity is associated with a lipid containing molecule chloroform methanol and acetone extraction methods were applied. Repeated experiments have shown the material to be unextractable, suggesting it not to be lipid containing molecules. Attempts to bind EDF(s) to affinity chromatography columns have proven unsuccessful. From ion exchange chromatographies, Q sepharose was found to bind the activity at pH 8.0 and even better at pH 9.0.

Biological Activity of EDF(s) Purified by Gel Filtration and Ion Exchange Chromatography

Separation of EDF(s) based on their molecular weight was done by a Sephacryl SF-300 column. Application of 10 ml of starting material, resulted in the separation of 3 distinct peaks displaying biological activity (data not shown). Activity, eluted from SF-300 column prior to chymotrypsinogen (a molecular weight marker), was estimated to be approximately 30 kd and designated as higher molecular weight EDF (EDF-H). The activity detected at the tail end of the

column was estimated to be approximately 1-2 kd and designated low molecular weight EDF (EDF-L). An additional peak of biological activity was detected and estimated to be approximately 5 kd. EDF-L and EDF-H were further purified and investigated. Fractions collected from SF-300 column and shown to have gel contraction inhibiting activity were pooled separately, based on their m.w., and applied to Q-Sepharose.

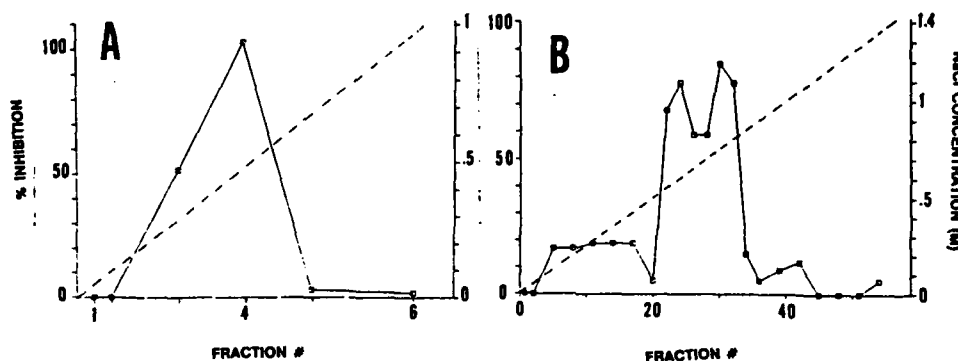


Fig. 1. Elution profile of EDF from ion exchange columns. (A) Q-Sepharose (B) FPLC Mono Q. The biological activity was detected by collagen contraction assay described in the text.

As shown in Fig 1A the elution of the biologically active EDF-L in a step-wise salt gradient was achieved at 0.5 M NaCl concentration. To concentrate the material and to purify it further, the pooled fractions were applied to an FPLC mono Q column (see Fig. 1B) and eluted with a linear gradient of 0.05-1.4 M NaCl. The biological activity was detected in fractions eluted with 0.6-0.8 M salt concentrations. EDF-H and EDF-L had a similar elution profile (data not shown). Following the above described purifications steps the inhibitory activity of EDF-H and EDF-L in the collagen gel contraction assay was the same. Serial dilutions indicated the presence of inhibitory activity in both at 1:80, but not at higher dilutions (data not shown). To evaluate whether the *in vitro* inhibitory effects of partially purified EDF(s) will translate into inhibition of wound contraction, they were applied to surgically-inflicted

(0.040 inch deep) wounds in pigs.

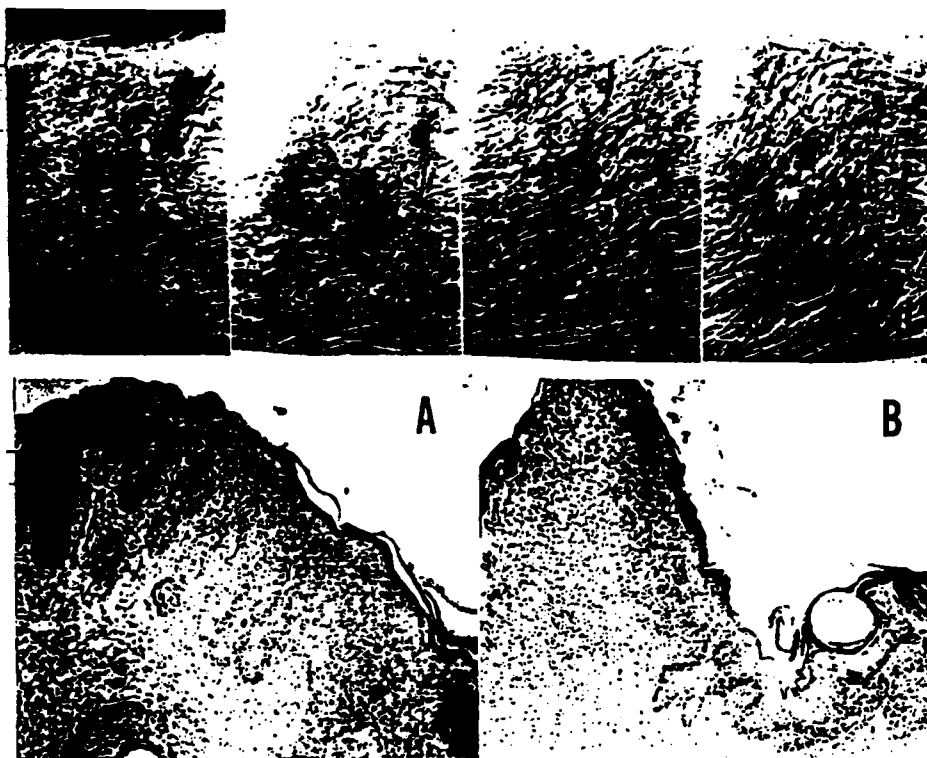


Fig. 2. The effects of EDF on wound healing (a-d). Clinical appearance of wound sites at 19 days, treated at time 0 with (a) EDF-L (1:8) ; (b) PBS; (c) Epidermal cell extract (1:400); (d) EDF-H (1:8); Note: The visible contraction of (b) in comparison of EDF treated wounds. (A) Histological appearance of EDF-L treated wound after 7 days, compared to (B) PBS control at the same time.

As documented in Fig 2., EDF(s) had striking effects on preventing wound contraction, when compared to control wounds treated with PBS. The difference was noticeable already 7 days after wounding but became more obvious at day 19. At this time the area resurfaced by new epithelium in wounds treated with EDF(s) was ~ 80% of the total surface area at time of wounding, compared to 64% in the control wounds treated with PBS. Consequently the PBS treated wound contracted twice as much as wounds treated with EDF. Contraction inhibiting activity of the epidermal cell extracts and partially purified EDF(s) appeared to be the same. The clinical data therefore suggested that in vitro collagen

contraction assay and in vivo wound contraction can be correlated. Evaluation of histological specimens taken 7 days after treatment of experimental wounds revealed that the density of fibroblasts in wounds treated with EDF(s) was ~ 1/10 of that found in wounds treated with PBS. In contrast to the fibroblast inhibiting activity EDF-L also had an epidermal cell growth promoting activity. As shown in Fig 2A, in wounds treated with EDF-L the rete pegs were deeper and epidermis covering the wound was much thicker than in the control (PBS) treated wounds (See Fig 2B). The activity of EDF-L and EDF-H on growth stimulation of a subpopulation of epidermal cells was also detected in studies in vitro.

DISCUSSION

The experiments described here reveal that the partially purified EDF-H and EDF-L have an inhibitory effect on the contractility of fibroblasts in vitro and wound contraction in vivo. EDF(s) in addition had a stimulatory effect on the growth of epidermal cells both in vitro and in vivo.

Previous clinical observations of wound healing suggested, that epidermis may regulate dermal activity. It was noted, that following injury, migration into wounded area and proliferation of fibroblasts ceased when wounds became resurfaced by functional epithelium (Rev. In Hunt et al, 1984). Moreover, substantial inhibition of wound contraction was observed in full thickness wounds transplanted with multilayered epidermal sheets grown in vitro (Eisinger, 1985). Thus, our findings of epidermal cell derived factors, shown to down regulate dermal activities, may furnish an explanation for previous clinical observations.

Throughout the process of purification of EDF(s), an in vitro assay of inhibition of contraction of three dimensional collagen gels was used. The assay was based on previous discovery that fibroblasts, in the presence of serum, in-

duce contraction of collagen gels (Bell et al, 1983). From known growth factors, TGF- β was shown to substitute for serum and markedly enhance the contraction normally observed in the presence of serum (Montesano, Orci, 1988). Under the same conditions platelet derived growth factor, basic fibroblast growth factor and epidermal growth factor did not significantly change contraction of collagen gels or collagen sponges (Eisinger et al, 1988a), suggesting that TGF- β is the most potent inducer and EDF an inhibitor of collagen contraction. The ability to contract a collagen matrix *in vitro* is believed to represent a fibroblast function that also operates *in vivo* during the process of wound repair (Ehrlich and Wyler, 1983). The cells responsible for contraction are the fibroblasts, particularly the so-called myofibroblasts characterized by myofibrils (Gabbiani et al, 1972). Wound contraction is of clinical importance, since it can reduce the amount of new tissue needed to reestablish organ integrity after tissue loss. However, this mechanism of wound closure is most desirable in mammals whose skin is loosely attached to underlying tissue. Human skin, with the exception of scalp, is closely knit to the tissues beneath it. Thus, wound contraction in human beings, if it occurred inappropriately, may lead to excessive scarring, constriction, immobilization and other disabilities.

We have shown good correlation between the *in vitro* inhibition of collagen gel contraction and inhibition of wound contraction *in vivo*, by EDF(s). Using both, *in vitro* and *in vivo* systems, studies toward understanding the mechanism of EDF(s) action can be initiated. Of particular interest will be to investigate whether EDF(s) can counteract TGF- β 's actions since both, *in vitro* and *in vivo* they elicit opposite biological effects. A prerequisite for detailed studies of EDF(s) is, however, the availability of sufficient amounts of purified factors.

The results presented here suggest, that more than one molecular form, found in epidermal cell

extracts, exhibits the EDF biological activity. Even though, EDF-L and EDF-H share most biochemical and biological properties, they differ in their molecular size. Therefore, possibilities, that the 1 Kd EDF is a degradation product of the 30 Kd EDF, or that the 30 Kd peak of activity results from aggregation of 1 or 5 Kd EDF(s), were considered. Use of inhibitors of proteolytic enzymes in the starting materials did not exclude the low molecular weight EDF(s). Similarly, treatment of 30 Kd EDF with different agents known to disaggregate proteins did not provide evidence for the second alternative. In view of current knowledge about other well studied growth factors, the finding of different molecular forms of EDF is not surprising. Particularly, members of TGF- β and b-FGF families were shown to vary in their size, but not necessarily in function (Wozney et al, 1988; Gospodarowicz et al, 1987).

Because of clinical desirability for negative dermal and positive epidermal regulatory factors, and their value as tools for studies of growth regulation, our efforts toward detailed characterization of EDF molecules will continue. Elucidation of the factor's characteristics and mode of action should be of value both in understanding the normal mechanism of wound healing and, ultimately, in controlling its abnormal or clinically undesirable manifestations.

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